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THE COUNCIL FOR TOBACCO RESEARCH–U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

RECEIVED
DEC 27 1973 Date:

1. Principal Investigator (give title and degrees): Herbert S. Rosenkranz, Ph.D.
Professor of Microbiology

2. Institution & address:
College of Physicians and Surgeons
Columbia University
630 West 168 Street
New York, New York 10032

3. Department(s) where research will be done or collaboration provided:

Department of Microbiology

4. Short title of study:

STUDY OF THE POTENTIAL MUTAGENICITY OF CIGARETTE SMOKE CONDENSATES

5. Proposed starting date: July 1, 1974

6. Estimated time to complete: 1 year

7. Brief description of specific research aims:

A) The mutagenicity of cigarette smoke extracts and fractions derived therefrom will be investigated. (Preliminary results indicate the presence of mutagens in such extracts).

B) Attempts will be made to identify the nature of the substance(s) giving a positive test.

C) The presence of mutagens in the urines of smokers (and non-smoker controls) will be investigated.

D) The ability of tissue extracts to enzymatically activate substances present in cigarette smoke extracts will be investigated.

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8. Brief statement of working hypothesis: It is well recognized that many known carcinogens are mutagens as well. Therefore screening for mutagens can be used to detect potential carcinogens.

The procedures for detecting mutagens are rapid, simple and relatively inexpensive. They are, therefore, well-suited for the project described herein.

9. Details of experimental design and procedures (append extra pages as necessary)

INTRODUCTION

The effects of environmental agents on human health is a subject of considerable concern at the present time (1, 2) especially as the use of chemical pollutants is on the increase (3-5). Of special concern to health scientists are the long-term effects of such environmental agents and more specifically their possible carcinogenicity, mutagenicity and teratogenicity. It has been estimated that at least 75% of all human cancers are due to environmental factors (6). Recently atmospheric extracts have been shown to be oncogenic in several systems (7-9, for additional references see ref. 7; for an analysis of the components of the atmosphere, see ref. 10). Ideally all environmental agents should be assayed in animals to determine their potential genetic and carcinogenic effects. Such an undertaking would however be time-consuming and very expensive and would possibly be beyond our national resources at the present time. Moreover to obtain reliable results that could be extrapolated to the human population, the number of animals required per group would have to be 30,000 rather than the 50 or 100 which are used currently (11). Furthermore, there is the added complicating factor of species specificity, agents affecting one species may have no effect on another. Thus 2-naphthylamine is a potent carcinogen

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9. (continued)

for man but it is without effect on the rodents commonly used in testing for carcinogenicity (12). Therefore, even if it were economically possible to test all environmental agents in animals, such a screening is not always foolproof. It has been suggested (1) that microbial systems capable of detecting mutagens and potential carcinogens be developed and used in screening. Indeed a national effort in this direction is currently being organized by the Division of Cancer Cause and Prevention of the National Cancer Institute and the applicant is a participant in that study. Substances that give positive tests in these microbial assays will then be tested in animals, while such tests are underway, the suspected agent could possibly be withdrawn from general use or its use could be regulated.

It is the applicant's experience that two microbial assays lend themselves to an investigation of the potential mutagenicity of substances present in tobacco smoke condensates. Indeed the applicant has obtained preliminary data suggesting the feasibility of such a study (see below). In view of the known relationship between mutagenicity and carcinogenicity (13-16), it is believed that results obtained by the procedures outlined will have a bearing on the health hazard associated with cigarette smoking.

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EXPERIMENTAL PROCEDURES

Mutagenic Effects:

A powerful system for detecting mutagens and carcinogens was introduced recently by Ames (17,18). The procedure is quite simple and involves placing the mutagen at the center of a Petri plate containing minimal medium that is seeded with bacteria (S. typhimurium) unable to grow because of a deficiency in their histidine biosynthetic pathway. Revertants to histidine-independence are seen as colonies in a ring around the area on which the agent has been deposited. After testing a large number of mutants, Ames selected several strains with low spontaneous

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reversion rates and high sensitivity to various agents. Each of the strains represents a different class of mutants: a) strains which detect mutagens that cause base-substitutions, they revert either by direct mutation or by suppressor mutations and b) strains capable of detecting frameshift mutations.

This procedure appears to be capable of detecting all sorts of mutations and it may well be the most comprehensive of the procedures available to date. This method has been used not only by Ames but by others as well to detect and identify environment mutagens and carcinogens (see for example refs. 17-25). A modification of the procedure in which the test substance is incorporated into a soft agar overlay has also been developed (19). The results obtained by this procedure are more quantitative since they do not depend upon the rate of diffusion of the test substance and it is especially useful for detecting poorly diffusible substances (25).

Microroganisms Deficient in DNA Repair

"Normal" cells exposed to noxious agents which alter the cellular DNA will attempt to overcome this effect by excising portions of the modified DNA and resynthesizing the correct sequence. The enzyme DNA polymerase I has been implicated in this repair process (both in the repair replication step and in the excision step in excision repair) (26-32). It is to be expected, therefore, that cells lacking this repair enzyme will be more sensitive to the action of agents which react with cellular DNA. The recent availability (27) of E. coli mutants (pol A_1^-) lacking this enzyme, has permitted verification of the prediction. Using normal (pol A^+) and DNA polymerase-deficient (pol A_1^-) strains of E. coli, it was shown that pol A_1^- was much more sensitive than pol A^+ to a large number of agents known to alter cellular DNA, these included known mutagens and carcinogens (27, 30, 31, 33-40).

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Based upon these observations a simple assay procedure was devised in this laboratory (38). Bacteria (pol A^+ and pol A_1^-) are spread onto the surface of agar plates and discs containing the substance to be tested are placed on the plates. After incubation (7-16 hours) the diameter (or areas) of the zones of inhibition are measured. Agents known to alter cellular DNA were found (38) to produce larger zones of inhibition on the pol A_1^- plates than on the corresponding pol A^+ ones. Agents known not to alter the cellular DNA (e.g. cycloserine, chloramphenicol, methicillin, etc.) gave equal zones of inhibition on both sets of plates. No conclusions could be drawn for substances which inhibited neither strains as this could be due either to inertness of the substance, or inability to penetrate into the cell or finally it could reflect the fact that the test substance normally requires metabolic activation but that this is beyond the metabolic machinery of the bacterium (but see below).

Using this procedure, the applicant and his collaborators (41-48), as well as others (49) tested a number of environmental agents some of which gave positive results. Since it is probable that intercalating agents (frameshift mutagens) are an important class (19) of environmental agents (e.g. polycyclic hydrocarbons intercalate in DNA (50)) it may be of significance that nitrosofluorene and N-hydroxylaminofluorene gave positive results in this system (38).

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Metabolic Activation of Mutagens and Carcinogens

In the bioassay system using E. coli pol A_1^- and Salmonella (described above) it was found that a number of known carcinogens (e.g. dimethylnitrosamine, diethylnitrosamine, 4-aminobiphenyl, etc. (25)) were completely inactive. Presumably this was a reflection of the fact that these substances are not the ultimate carcinogens and they require metabolic activation by mammalian enzymes (51-56). Such activation is beyond the metabolic capability of microorganisms (57).

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To overcome these problems extracts from rat liver with certain added co-factors were incorporated into the plate assays and this permitted the determination of the preferential inhibition of the pol A_1^- by dialkylnitrosamine (38) as well as by a number of aromatic amines (25). More recently it was found that better results could be obtained if a mixed extract derived from rat and hamster livers was used (25). A similar modification (16) of the mutagenesis assay using Salmonella has permitted the demonstration of the mutagenicity of a number of carcinogens which are known to require metabolic activation (16,25).

PRELIMINARY FINDINGS:

A series of cigarette smoke condensates as well as fractions derived therefrom were kindly provided by the Agricultural Research Service. Upon analysis in the DNA polymerase-deficient (pol A_1^-) E. coli system it was found that some of these substances preferentially inhibited the growth of the pol A_1^- strain (Table 1). This is a property they shared with the known mutagens and carcinogens propane sultone, methylnitrosonitroguanidine, β-propiolactone, 4-hydroxylaminoquinoline-N-Oxide (Table 1) and others. Agents known to react with cellular sites other than the DNA inhibited the parent (pol A^+) and pol A_1^- strains to the same extent; e.g. chloramphenicol, methicillin and streptomycin (Table 1).

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The effect of the cigarette smoke condensates on the Salmonella mutagenesis system was also investigated. The procedure for detecting revertants to histidine-independence was discussed above. The Salmonella typhimurium strains used TA 1530, TA 1535 and TA 1538 were kindly provided by Dr. Bruce N. Ames. Strains TA 1530 and TA 1535 are essentially isogenic, they have a base-pair change in the histidine G gene and can be used to detect mutagens causing base-pair

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Table 1

Effect of Cigarette Smoke Condensates on DNA Polymerase-Deficient Bacteria

Substance	Amount (mg)	Diameters of Zones of Inhibition	
		Pol A^+	Pol A_1^-
Crude Tar	2.6	8.1	11.6
Benzene Extract	1.3	0(6.35)	8.0
Fraction No. 2	5.3	11.1	15.2
Fraction No. 9	1.5	7.8	11.1
Fraction No. 13	3.8	8.3	13.7
Propane sultone	250 µg	11.9	18.9
N-Methyl-N-Nitroso-N'-Nitroguanidine	250 µg	25.0	33.8
β-Propiolactone	2 µl	12.3	20.2
4-Hydroxylaminoquinoline-N-Oxide	250 µg	7.7	11.1
Chloramphenicol	30 µg	28	28
Methicillin	30 µg	29	29
Streptomycin	10 µg	19	19

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changes. However strain TA 1535 differs from TA 1530 in that it is a deep-rough strain deficient in cell envelope lipopolysaccharides which increases its permeability to large molecules. Strain TA 1538 has a mutation in the D gene of the histidine operon, it is also a deep-rough strain. It can be used to detect frameshift mutations. In the assay that was used known quantities of test substances were incorporated into the agar overlay together with the test bacteria.

Our preliminary results, which are the averages of duplicate determinations indicate that the cigarette smoke extracts specifically increased the rate of mutation of strain 1538 (Table 2). This is taken to mean that the cigarette smoke extracts contained mutagens which caused frameshift (but not base-substitution) mutations. Frameshift mutations are generally caused by planar molecules capable of intercalating between base-pairs of the DNA double-helix. This leads to the prediction that the substance(s) responsible for the positive test may be aromatic in character. (The reliability of the assay was tested by using methylmethanesulfonate (MMS) and 4-hydroxylaminoquinoline-N-Oxide (HAQNO) as controls (Table 2). The former causes only base-substitutions while the latter induces frameshift mutations. In fulfillment of this prediction it was found that MMS induced mutations only in strains TA 1530 and TA 1535 while HAQNO mutated strain TA 1538 only (Table 2)).

RESEARCH PROPOSAL

I. The results summarized in Tables 1 and 2 are very promising and they certainly warrant repetition. It is proposed, therefore, to repeat the assays using a new batch of cigarette smoke extracts and fractions derived therefrom.

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Table 2

INDICATION OF POSSIBLE MUTAGENICITY OF CIGARETTE SMOKE CONDENSATES

Fraction	µg	Revertants per Plate		
		TA 1530	TA 1535	TA 1538
Control (Acetone)	0	21	27	11
CRUDE TAR	26.4	22	20	15
Benzene Extract	13.0	24	27	26
Fraction No. 2	52.5	23	24	25
Fraction No. 9	15.3	22	24	23
Fraction No. 13	38.2	22	22	16
Fraction No. 14	0.6	22	22	16
Methylmethanesulfonate	10 µl	63	41	10
4-Hydroxylaminoquinoline-N-Oxide	1 µg	17	20	40
Water	10 µl	20	22	11

Cigarette products and the other test substances were incorporated into the soft agar layer and plates incubated in the dark for 48 hours.

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The data of Table 2 suggest that there is some specificity to the substance(s) responsible for the mutagenicity, i.e. only the strain indicative of frameshift mutations was affected. However due to a shortage of material, only low levels of cigarette smoke extracts could be tested and accordingly the number of mutants obtained was small. When more material becomes available the assay will be repeated. This should enable us to get more meaningful numbers and to ascertain whether other types of mutations (i.e. base-substitutions) can be detected when the amount of test material is increased.

II. Should, as expected, the above experiments be reproducible, then it will be possible to test cigarette smoke extracts derived from a variety of tobaccos and also from different parts of the tobacco plants.

III. As has been mentioned above, the bioassays used are simple and rapid. It is feasible therefore to use them to monitor the isolation of the active principle. Accordingly, efforts will be made to purify the activity principle by solvent extraction and chromatographic procedures in order to establish the nature of the active principle.

IV. Many carcinogens require metabolic activation (see above) prior to exhibiting biologic activity in microbial systems. This ability to activate carcinogens is beyond the metabolic activity of bacteria. However by incorporating a mammalian cell-free extract into either the E. coli or the Salmonella system it is possible to activate these substances. Thus for example neither 4-aminobiphenyl nor 2-fluorenamine exhibit appreciable activity in the Salmonella system, yet when a rat liver extract is incorporated into the assay there is a tremendous increase in mutagenic activity (Table 3). It should be noted that the increase in mutagenic activity is specific for one strain only (Table 3).

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TABLE 3

responsible for the mutagenicity, i.e. only the strain indicative of frameshift mutation

Salmonella Mutagenesis: EFFECT OF ACTIVATION *

Substance	Amount	Revertants per Plate					
		No Activation			With Activation		
		1530	1535	1538	1530	1535	1538
4-Aminobiphenyl	250 µg	22	34	13	27	33	140
2-Fluorenamine	250 µg	10	18	106	30	33	5655

mutant obtained was small. When

*The test substance as well as the liver extract and required co-factors were incorporated into the soft agar overlay.

It is proposed, therefore, to determine whether the activity of cigarette smoke extracts in the E. coli and Salmonella system can be increased (or the specificity altered) by incubation in the presence of cell-free extracts derived from various tissues. Such a study should indicate which organ, if any, is involved in the metabolic conversion of tobacco derivatives.

V. Patients receiving cytoxan (cyclophosphamide) - therapy excrete a mutagen (an activated alkylating agent) in their urine. The activity of this substance can be demonstrated by the microbial assay procedures described above. It is proposed, therefore, to test the urines of heavy smokers (as well as of non-smoker controls) for the presence of a substance giving a positive test in the microbial bioassay systems. (Of course, care will be taken to select only individuals not receiving any medication and who are otherwise healthy). It may be necessary to concentrate the urines prior to testing. However, should positive urines be obtained, then the bioassay can be used to monitor the isolation of the active principle.

Significance of this Research:

The outline described above should help determine whether tobacco

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smoke condensates contain (a) substance(s) endowed with mutagenic (and therefore carcinogen) potential. If such (a) substance(s) exist(s) then the procedures described herein may help in identifying them and determining their chemical nature.

The proposed investigation may establish if the potential mutagenicity of cigarette smoke extracts is enhanced by cellular metabolic events and if so which organ is involved.

If the mutagenic agent is present in the urine of smokers, then it may be possible to correlate the amount of this material present to a) quantity of cigarettes consumed, b) the type of cigarette smoked, c) age, d) sex, e) state of health, medication taken, etc. The assay could also be used to determine for how long this substance is excreted after smoking is stopped. Obviously, many of these subsequent studies depend upon the earlier findings.

It is conceivable that these investigations could lead to the development of a "safer cigarette"; perhaps certain conditions will lead to a tobacco free of the mutagen. Moreover if the properties of the active principle are determined, it may be selectively extracted from the tobacco.

Experience of the Principal Investigator:

The applicant has first-hand knowledge of all the procedures described herein. The systems described are all in operation in his laboratory. A detailed curriculum vitae and a list of publications is appended hereunto.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The laboratories in which these studies will be carried out are in the William Black Research Building, College of Physicians and Surgeons, Columbia University. These laboratories are equipped to carry out all of the procedures required for the completion of this research proposal. In addition, the Department of Microbiology, Columbia University, has good equipment for spectroscopic and radioisotopic determinations. The Department maintains an amino acid analyser, and an analytical ultracentrifuge equipped with Schlieren, ultraviolet and optical systems, microdensitometers, fluorescence microscopes, electron microscopes and accessory equipment, preparative centrifuges, constant temperature rooms and equipment for various chromatographic procedures. Facilities for tissue culture and bacteriological work are also available.

11. Additional facilities required:

NONE

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12. Biographical sketches of investigator(s) and other professional personnel (append):

Appended

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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14. First year budget:

A. Salaries (give names or state "to be recruited") Professional (give % time of investigator(s) even if no salary requested)		% time	Amount
Herbert S. Rosenkranz,	Principal Investigator	20	—
William T. Speck,	Co-Investigator	20	—
Technical			
1 Technician	"to be recruited"	100	8,500
Fringe Benefits			1,190
		Sub-Total for A	9,690
B. Consumable supplies (by major categories)			
Glassware and media			1,600
		Sub-Total for B	1,600
C. Other expenses (itemize)			
Maintenance of Equipment			400
Cost of Publishing (Reprints, etc)			400
Office Supplies			200
		Sub-Total for C	1,000
		Running Total of A + B + C	12,290
D. Permanent equipment (itemize)			
None			—
		Sub-Total for D	
E. Indirect costs (15% of A+B+C)		E	1,843.50
		Total request	14,133.50

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	$10,270	1,600	1,000	—	1,931	$14,801
Year 3						

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16. Other sources of financial support:
List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
In vitro and host-mediated Microbial Mutagenicity Assay	NCI	$65,135	June 1973-June 1974
Research Career Development Award	NIH	Salary of	Principal Investigator 1965-1975
Gift	Marion Laboratories	$27,000	per year
Gift	G.A. Carden Special Fund for Cancer Research	4,000	Unlimited

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Role of RNA in DNA Viruses	N.I.H.	$20,000	?

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Herbert S. Rosenkranz

Signature Herbert S. Rosenkranz Date 11/21/73

Telephone 212 (Area Code) 579-3658 (Number) (Extension)

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Columbia University

Mailing address for checks
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Assistant Vice President in
Charge of Medical Affairs

Responsible officer of institution

Typed Name Frederick B. Putney, Ph.D.

Title Assistant Vice President in Charge of Medical Affairs

Signature Frederick B. Putney Date 11/21/73

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